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CORRESPONDENCE

Research Correspondence

The Vascular Smooth Muscle Cells Apoptosis in Asymptomatic Diabetic Carotid Plaques: Role of Glycemic Control

To the Editor: Atherosclerotic plaque instability is enhanced in diabetes, and it determines the increased incidence and severity of clinical events (1). A variety of proinflammatory cytokines, including tumor necrosis factor (TNF)-9 and interleukin (IL)-1β, alone or in conjunction have the potential to promote plaque instability, inducing apoptosis (2). Vascular smooth muscle cell (VSMC) apoptosis has been identified in advanced human atherosclerotic plaques and is increased in unstable versus stable lesions (3). Although it has been demonstrated that diabetes mellitus may enhance apoptosis in myocardial (4) and endothelial cells (5), still, no evidence exists in humans about the potential role of diabetes in the evolution of diabetic atherosclerotic plaques toward instability by enhancing VSMC apoptosis. We hypothesize that by enhancing the proinflammatory cytokines, diabetes may exert proapoptotic effects on VSMCs of atherosclerotic plaque. To address these issues, cell death by apoptosis and expression and localization of TNF-9 and IL-1β was evaluated in carotid plaques of asymptomatic diabetic and nondiabetic patients.

The study group consisted of 26 type 2 diabetic and 30 nondiabetic patients enlisted to undergo carotid endarterectomy for asymptomatic extracranial high-grade (>70%) internal carotid artery stenosis (6). Written informed consent was obtained from all patients. The local ethics review committee approved the study. After surgery, the specimens were cut perpendicular to the long axis into two halves. The first half was frozen in liquid nitrogen, lysed, and centrifuged for the following enzyme-linked immunosorbent assay analysis. Caspase-3, TNF-α, IL-1β and nitrotyrosine levels were quantified in plaques using specific kits (R&D Systems, Minneapolis, Minnesota; Imgenex, Oxford, United Kingdom). A portion of the other half specimen was immediately immersion-fixed in 10% buffered formalin. Serial sections were incubated with specific antibodies α-smooth muscle (SM) actin and anti-CD68; anti–TNF-α, IL-1β and active caspase-3 as well as terminal deoxynucleotidyl transferase end-labeling (TUNEL).

Clinical data for the study population are presented in Table 1. In nondiabetic subjects, the presence of type 2 diabetes mellitus or impaired glucose tolerance was tested by oral glucose tolerance test. Compared with nondiabetic patients, diabetic patients had significantly greater portion of plaque area occupied by macrophages (24.5 ± 5% vs. 5 ± 2% of the total area; $p < 0.01$) (Fig. 1) and lower content of interstitial collagen ($p < 0.01$) (Fig. 1). The nondiabetic plaques were composed primarily of longitudinally oriented SMCs that strongly expressed α-SMC actin (18 ± 6% of the total area). However, α-SMC immunoreactivity was much lower in the diabetic plaques (9 ± 7% of the total area) (Fig. 1). We detected IL-1β and TNF-α in 22 of 26 diabetic plaques, whereas the same cytokines were detected in only 12 of 30 nondiabetic plaques. Compared with nondiabetic lesions, diabetic plaques had higher levels of IL-1β and TNF-α ($p < 0.01$) (Fig. 1). Notably, IL-1β and TNF-α plaque levels were strongly dependent on glycemic control, as also reflected by the statistically significant correlation between plasma HbA1c and cytokine plaque levels (IL-1β: $r = 0.49$, $p < 0.001$; TNF-α: $r = 0.51$, $p < 0.001$). The TUNEL labeling disclosed evidence of apoptosis in 32 (57%) of

Table 1. Characteristics of Study Patients

Variables	Diabetic Patients (n = 26)	Nondiabetic Patients (n = 30)
Age (yrs)	68 ± 3	69 ± 2
Men/women	15/11	16/14
Patient characteristics		
Family history of IHD	15	14
Hypertension	11	15
Hypercholesterolemia	10	13
Cigarette smoking	7	12
Coronary artery disease	14	13
BMI (kg/m^2)	28.2 ± 2	27.6 ± 2
Systolic blood pressure (mm Hg)	129 ± 11	128 ± 12
Diastolic blood pressure (mm Hg)	81 ± 4	81 ± 4
HbA1c (%)	8.1 ± 1.4	4.9 ± 1.1*
Fasting blood glucose (mmol/l)	9.7 ± 1.4	6 ± 0.9*
Insulin (μU/ml)	10.6 ± 2.4	8.06 ± 3.3*
Total cholesterol (mmol/l)	5.53 ± 0.03	5.39 ± 0.06
HDL cholesterol (mmol/l)	1.22 ± 0.03	1.29 ± 0.04
Triglycerides (mmol/l)	2.31 ± 0.11	2.21 ± 0.12
Stenosis severity (%)	76.4 ± 4.5	75.5 ± 4.9
Percentage of macrophage-rich areas	24 ± 5	5 ± 2*
No. of T cells per mm^2 section area	70 ± 13	15 ± 7*
Percentage of expression of HLA-DR	21 ± 6	13 ± 7*
Percentage of VSMC-rich areas	9 ± 7	18 ± 6
Active therapy		
Aspirin	21	24
Warfarin	1	2
Beta-blocker	5	7
Calcium channel blocker	4	3
Statin	17	20
ACE inhibitor	14	16
Diuretic agent	6	9
AT-2 antagonist	12	14
Insulin	5	—
Sulfonylureas	20	—
Thiazolidinediones	1	—
Metformin	14	—

Data are presented as n or mean ± SD. HbA1c normal values: 4% to 6.2%. *$p < 0.05$ compared with diabetic group. Continuous variables were compared among the groups of patients with an independent Student t test for normally distributed data and Wilcoxon test for non-normally distributed data. The Kolmogorov-Smirnov test was used to assess whether continuous variables were normally distributed. We compared data using a Wilcoxon test for nitrotyrosine, interleukin-1, tumor necrosis factor-α, triglycerides, and insulin. Differences between measured variables were considered significant if the resultant p value was 0.05 or less. The strength of the association of plasma HbA1c with caspase-3 and cytokine levels in plaque specimens was assessed by linear regression analysis. The influence of HbA1c on plaque features was evaluated using multivariate analysis. All calculations were performed using the computer program SPSS 12.

ACE = angiotensin-converting enzyme; BMI = body mass index; HDL = high-density lipoprotein; IHD = ischemic heart disease; VSMC = vascular smooth muscle cell.

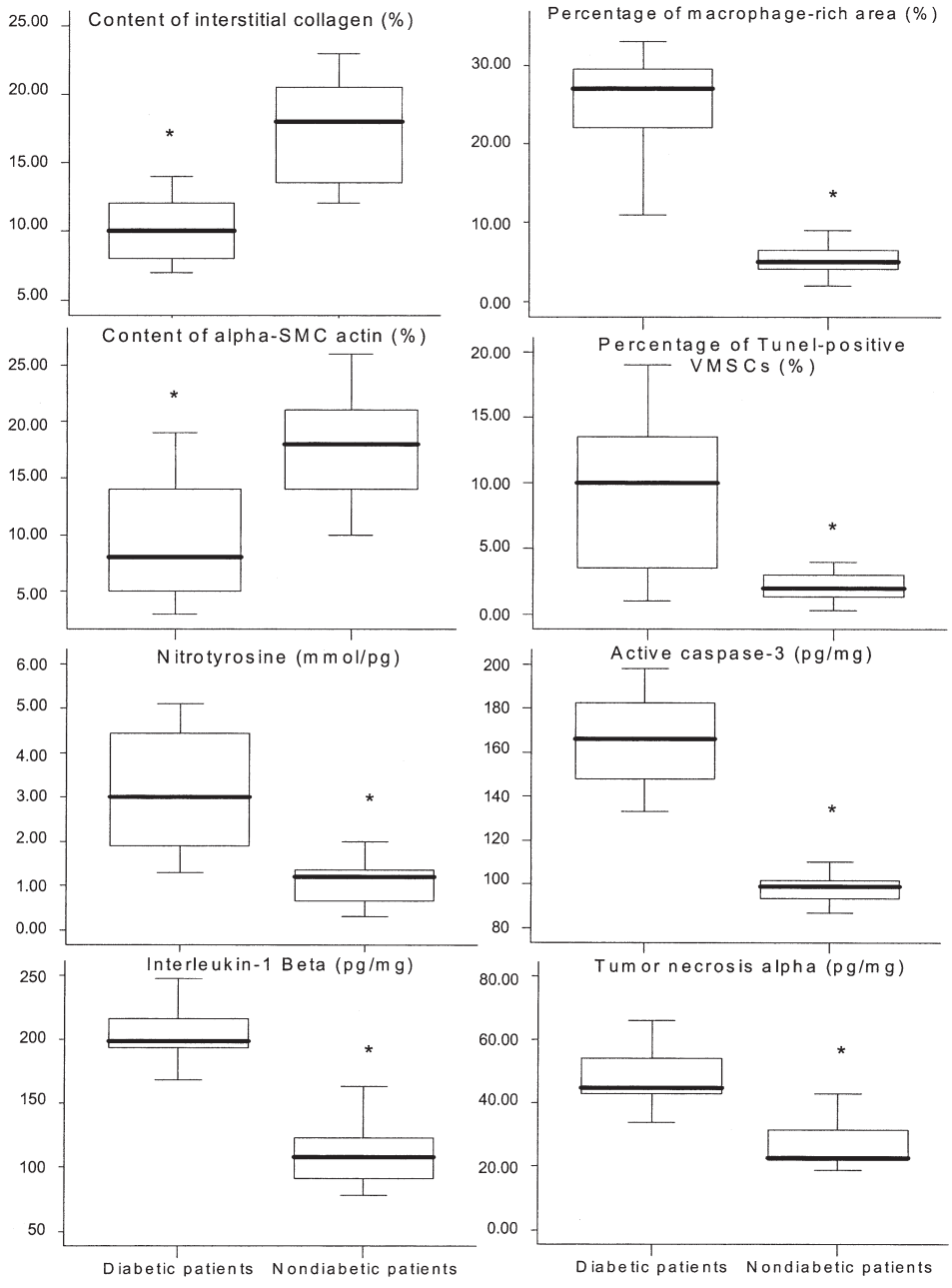

Figure 1. Percentage of interstitial collagen (Sirius red staining for collagen content), macrophage-rich areas, α-smooth muscle cell (SMC) actin and terminal deoxynucleotidyl transferase end-labeling (TUNEL)-positive vascular smooth muscle cells (VSMCs) in diabetic and nondiabetic plaques (to identify vascular smooth muscle cells undergoing apoptosis, double staining was performed with TUNEL and periodic acid-Schiff staining or TUNEL and α-smooth muscle actin staining). The **bars** show levels of nitrotyrosine, active caspase-3, interleukin-1β, and tumor necrosis factor-α in diabetic and nondiabetic plaques (The **central line** represents the median, the **boxes** span from the 25th to 75th percentiles, and the **error bars** extend from the 10th to 90th percentiles). *$p < 0.05$ compared with nondiabetic patients.

the total 56 specimens studied. Among 26 diabetic plaques, 24 (92%) contained foci of apoptosis; in contrast, apoptosis was observed in only 8 (27%) of 30 nondiabetic plaques ($p < 0.01$). Among nondiabetic plaques, apoptosis was typically limited to <2% of cells. Among diabetic plaques, the frequency of apoptotic cells ranged from 0.10% to 18% (4.9 ± 6.5%). Compared with nondiabetic plaques, diabetic plaques revealed higher levels of caspase-3 ($p < 0.01$) (Fig. 1). Notably, caspase-3 levels in plaque specimens were strongly dependent on glycemic control ($r = 0.533$, $p < 0.001$). Diabetic plaques had significantly higher percentage of TUNEL-positive VSMCs compared with nondiabetic plaques ($p < 0.01$) (Fig. 1). Higher nitrotyrosine levels were found in diabetic as compared with nondiabetic plaques ($p < 0.001$) (Fig. 1). Most TUNEL-positive diabetic plaques (75%) showed higher levels of TNF-α and nitrotyrosine levels. Conversely, most fields that were not

TUNEL-positive (72%) showed moderate to low levels of TNF-α and nitrotyrosine levels. Moreover, by multivariate analysis, HbA1c was independently related to VSMC apoptosis, as showed by TUNEL-positive VSMCs and caspase-3 levels ($p < 0.030$ and $p < 0.026$, respectively).

Our study shows that VSMC apoptosis may be involved in the evolution of diabetic plaques toward instability. In particular, VSMC apoptosis is greater in diabetic lesions than nondiabetic lesions and was associated with higher TNF-α and IL-1β levels along with lower interstitial collagen and α-SMC actin content. All this would make diabetic plaques more prone to inflammatory-dependent rupture and might increase the risk of cerebrovascular ischemic events (7,8). In our study, macrophages were more abundant in diabetic plaques and represented the major source of inflammatory cytokines, suggesting the presence of an active inflammatory reaction in diabetic plaques. In agreement with the difference in apoptosis staining pattern, the histologic milieu of the lesions appears different with regard to cellularity, but not in the degree of vessel stenosis, suggesting that diabetic and nondiabetic lesions are different only with regard to inflammatory burden. Hence, the differences in plaque behavior likely stem from differences in the presence of stimuli (i.e., persistent hyperglycemia and oxidative stress, as evidenced by high HbA1c and nitrotyrosine levels) for selective expression of TNF-α or IL-1β capable of disrupting plaque stability via VSMC apoptosis. Notably, the intriguing and novel proatherogenic mechanism of apoptosis in human diabetes is supported in this study not only by the observation that VSMC apoptosis is higher in diabetic plaques, but also by the information that it is strongly correlated with the intensity of glycemic control as reflected by HbA1c. The mechanism of the relationship of cytokines with the VSMC apoptotic process remains uncertain in diabetic plaques. However, TNF-α and IL-1β do not act in isolation, as macrophage-induced VSMC apoptosis through formation of peroxynitrite also requires nitric oxide and oxygen free radicals (9). This suggests that cooperative interactions might occur between these mediators, and the present study outlines such associations demonstrating a presence of overproduction of nitrotyrosine, a good marker of peroxynitrite formation (10), in diabetic plaques that was not present in nondiabetic lesions. Indeed, these stimuli of diabetic milieu can also induce apoptosis of endothelial cells, resulting in vascular leak and inflammation, which are implicated in the pathogenesis of vascular diseases (3). This study demonstrates enhanced VSMC apoptosis in diabetic carotid atherosclerotic lesions and provides evidence that the activation of this mechanism by inflammatory cells is associated with an increase in oxidative stress potentially promoting plaque rupture. These findings are potentially important from a fundamental standpoint, because they indicate a pathogenetic role for the VSMC apoptosis in the evolution of diabetic atherosclerotic lesions. From a practical standpoint, these findings provide further support for the role of glycemic control on plaque stabilization in diabetic patients with atherosclerotic disease.

***Raffaele Marfella, MD, PhD**

*Department of Geriatrics and Metabolic Diseases
Second University of Naples
Naples
Italy
E-mail: raffaele.marfella@unina2.it

Clara Di Filippo, MD
Alfonso Baldi, MD
Mario Siniscalchi, MD, PhD
Ferdinando Carlo Sasso, MD, PhD
Basilio Crescenzi, MD
Francesca Cirillo, MD
Giovanni Francesco Nicoletti, MD
Francesco D'Andrea, MD
Gisberta Chiorazzo, MD
Emilio Musacchio, MD
Francesco Rossi, MD
Mario Verza, MD
Ludovico Coppola, MD
Michele D'Amico, MD

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Evidence of Cocaine-Related Coronary Atherosclerosis in Young Patients With Myocardial Infarction

To the Editor: Cocaine use has increased in the last years reaching in 1999 in the U.S. 30% of all drug-related visits to the emergency department, exceeding morphine and representing the most frequent cause of drug-related deaths (1). One of every four myocardial infarctions (MIs) in people aged 18 to 45 years can be linked to cocaine use (2). Several reports (3) implicated coronary vaso-